Apoptosis regulated by a death factor and its receptor: Fas ligand and Fas

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SUMMARY

Homeostasis in vertebrates is tightly regulated by not only proliferation and differentiation of cells, but also cell death or apoptosis (Ellis *et al.* 1991; Raff 1992). Many cytokines bind to their respective receptors to regulate proliferation and differentiation of cells. Our recent studies on the Fas ligand and Fas indicate that they work respectively as a death factor and its receptor and suggest that, in some cases, cell death or apoptosis is regulated by cytokines and their receptors. Here, I present the summary of the Fas/Fas ligand system which has been studied in my laboratory over the past 5 years, and I will discuss its physiological roles.

1. FAS, A RECEPTOR FOR A DEATH FACTOR

In 1989, two groups reported mouse monoclonal antibodies having a cytolytic activity on some human cells (Trauth *et al.* 1989; Yonehara *et al.* 1989). The antigens recognized by these antibodies were designated as Fas antigen (Fas) or APO-1. To assess the function of Fas, we isolated human and mouse Fas cDNAs (Itoh *et al.* 1991; Watanabe-Fukunaga *et al.* 1992*b*). Fas consists of 325 (human) or 306 (mouse) amino acids with a signal sequence at the N-terminus and a transmembrane domain in the middle of the molecule. The subsequent purification of human APO-1 and its molecular cloning (Oehm *et al.* 1992) established its identity with Fas. Fas is a member of the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor family (Itoh *et al.* 1991; Nagata 1993), which include Fas, two TNF receptors (types I and II), the low-affinity NGF receptor, B cell antigen CD40, T cell antigen OX40, CD27 and 4-1BB, and Hodgkin's lymphoma cell surface antigen CD30 (figure 1). The extracellular regions of the family members are rich in cysteine residues, and can be divided into three to six subdomains. The extracellular region is relatively conserved among members (about 24–30% identity), whereas the cytoplasmic region is not, except for some similarity between Fas and the TNF type I receptor (Itoh *et al.* 1991).

The thymus, heart, liver and ovary abundantly express Fas mRNA (Watanabe-Fukunaga *et al.* 1992*b*). Flow cytometry analysis using anti-Fas antibody indicated that most thymocytes except for double negative ($CD4^-$ $CD8^-$) thymocytes express Fas (Drappa *et al.* 1993; Ogasawara *et al.* 1993). Activated human T cells and B cells express Fas (Trauth *et al.* 1989), and lymphoblastoid cells transformed with human T cell leukemia virus (HTLV)-I, human immunodeficiency virus (HIV) or Epstein-Barr virus (EBV) highly express Fas (Nagata 1994). Some other tumour cell lines such as human myeloid leukemia, human squamous carcinoma and mouse macrophage cell lines also express Fas, although the expression level is low compared with that of the lymphoblastoid cells. Expression of Fas is up-regulated by interferon γ (IFN-γ) in mouse macrophage BAM3 and fibroblasts L929 cells, or human adenocarcinoma HT-29, or by a combination of IFN-γ and TNFα in human tonsillar B cells.

2. MUTATION OF FAS IN *lpr*-MICE

There is a single gene for Fas in human and mouse chromosomes (Adachi *et al.* 1993). The human Fas gene is located on chromosome 10q24.1 (Inazawa *et al.* 1992), whereas mouse Fas gene is in the region of chromosome 19, which is homologous to human 10q24.1 (Watanabe-Fukunaga *et al.* 1992*b*). Mouse Fas chromosomal gene consists of more than 70 kb, and is split by 9 exons (R. Watanabe-Fukunaga and S. Nagata, unpublished results).

Referring the location of Fas gene to the mouse Genomic Database (GBASE). Fas gene was found to be close to the locus called *lpr* (lymphoproliferation) (Watanabe *et al.* 1991). There are two allelic mutations, *lpr* and lpr^{cg}. The *lpr*-mice hardly express Fas mRNA in the thymus and liver (Watanabe-Fukunaga *et al.* 1992*a*). Accordingly, flow cytometry could not detect Fas on thymocytes from *lpr*-mice (Drappa *et al.* 1993; Ogasawara *et al.* 1993). A comparison of the Fas gene from *lpr*-mice with that of wild-type mice indicated an insertion of an early transposable element (ETn) in intron 2 of Fas gene (Adachi *et al.* 1993). ETn is a mouse endogenous retrovirus, of which about 1000 copies can be found in

Phil. Trans. R. Soc. Lond. B (1994) 345, 281–287
Printed in Great Britain

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2. MUTATION OF FAS IN *lpr*-MICE

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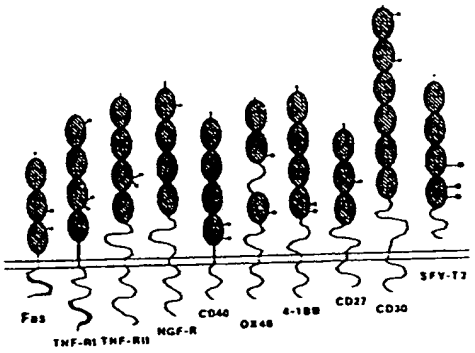

Figure 1. TNF/NGF receptor family. Members of the TNF/NGF receptor family are schematically shown. These include Fas, TNF type I and II receptors, low-affinity NGF receptor, CD40, OX40, 4-1BB, CD27, CD30, and the soluble protein coded by Shope fibroma virus. The slashed regions represent cysteine-rich subdomains. A domain of about 70 amino acids in the cytoplasmic regions of Fas and the type I TNF receptor has some similarity, and it is shown as a bold line. —● indicates N-glycosylation sites.

the mouse genome (Brulet *et al.* 1983). The ETn carries long terminal repeat (LTR) sequences at both 5′ and 3′ termini, which contains a poly(A) adenylation signal (AATAAA). Inserting the ETn into intron of a mammalian expression vector dramatically, but not completely, reduced the expression efficiency (Adachi *et al.* 1993). These results indicate that in *lpr*-mice, an insertion of an ETn into intron of Fas gene greatly reduces its expression, but this mutation is leaky. In contrast to the *lpr*-mice, lpr^{cg} mice express Fas mRNA of normal size as abundantly as the wild-type (Watanabe-Fukunaga *et al.* 1992*a*). However, this mRNA carries a point mutation, which causes a replacement of isoleucine with asparagine in the Fas cytoplasmic region and abolishes the ability of Fas to transduce the apoptotic signal (Watanabe-Fukunaga *et al.* 1992*a*).

3. FAS-MEDIATED APOPTOSIS

(*a*) *Apoptosis* in vitro

To assess the function of Fas, mouse cell transformants expressing human Fas were established using various mouse cell lines as host (Itoh *et al.* 1991). When the mouse cells expressing human Fas were treated with anti-human Fas antibody, they died within 5 h. Examination of the dying cells under electron microscope revealed extensive condensation and fragmentation of the nuclei, which is characteristic of apoptosis. Chromosomal DNA degraded in a laddered fashion after a 2 h incubation with the anti-Fas antibody. The human Fas expression plasmid has also been introduced into a mouse IL-3 (interleukin-3)-dependent myeloid leukemia FDC-P1 cell line (Itoh *et al.* 1993). Although the transformed cells died due to IL-3 depletion, they did so over 36 h, as observed with the parental cells. On the other hand, exposure to the anti-human Fas antibody killed the cells within 5 h in the presence of IL-3. From these results, we concluded that Fas actively transduces the apoptotic signal, and the cytolytic anti-human Fas antibody works as agonist.

(*b*) *Apoptosis* in vivo

We established hamster monoclonal antibodies against mouse Fas, which have cytolytic activity (Ogasawara *et al.* 1993). When this antibody is injected intraperitoneally into mice, the wild-type, but neither *lpr* nor lpr^{cg}, mice died within 5–6 h. These results indicate that the lethal effect of the anti-Fas antibody is due to binding of the antibody to the functional Fas in the tissues. The fact that lpr^{cg} mice expressing the non-functional Fas are resistant to the lethal effect of the antibody indicates little involvement of the complement system in this killing process. Biochemical analysis of sera showed a specific and dramatic increase of GOT (glutamic oxaloacetic transaminase) and GPT (glutamic pyruvic transaminase) level shortly after injection of the antibody, suggesting the liver injury. Histological analysis indicated focal hemorrhage and necrosis in the liver, whereas dying hepatocytes showed a morphology characteristic of apoptosis under electron microscope (figure 2). These results indicate that individual hepatocytes died by apoptosis. However, as it occurred so rapidly and so widely; granulocytes and macrophages could not phagocytose the apoptotic cells, and the tissues went to the secondary necrosis.

(*c*) *Apoptotic signal mediated by Fas*

The apoptotic signal through Fas is induced by binding of anti-Fas or anti-APO1 antibody, or the Fas ligand to Fas. The anti-human Fas antibody is an IgM antibody, whereas the anti-APO1 antibody is an IgG_3 antibody which tends to aggregate. The $F(ab')_2$ fragment or other isotypes of the anti-APO1 antibody hardly induces apoptosis (Dhein *et al.* 1992). On the other hand, the cytotoxic activity of the inactive anti-APO1 antibody can be reconstituted by cross-linking the antigen with a secondary antibody or with protein A. These results indicate that oligomerization of at least three Fas molecules is a biologically relevant complex in generating an intracellular signal. As described below, the fact that Fas ligand is a TNF-related molecule which exists as a trimer (Smith & Baglioni 1987), agrees with this hypothesis.

Activation of Fas induces degradation of chromosomal DNA within 3 h, which eventually kills the cells, suggesting that a strong death signal is transduced from Fas. In some cells, activation of Fas alone is not sufficient to induce apoptotic signal. The presence of metabolic inhibitors such as cycloheximide or actinomycin D is required to induce the Fas-dependent apoptosis in these cells (Itoh *et al.* 1991). On the other hand, the activated or transformed T cells can be killed by anti-Fas antibody alone. These results indicate that the signal-transducing machinery for Fas-induced apoptosis is present in most cells, and some cells express

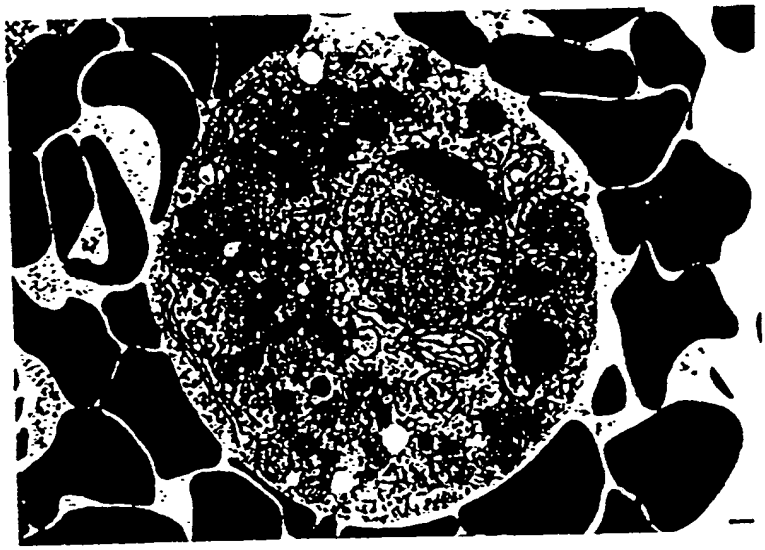

Figure 2. Fas-mediated apoptosis of hepatocytes *in vivo*. The purified anti-mouse Fas antibody (100 μg) was subcutaneously injected into mice and a liver section was examined under a transmission electron microscope. The affected hepatocytes show the condensed and fragmented nuclei characteristic of apoptosis.

a protein(s) which works inhibitory for Fas-mediated apoptosis. In fact, overexpression of oncogene Bcl-2 product which inhibits apoptosis in various system (Korsmeyer 1992) partly inhibited Fas-mediated apoptosis (Itoh *et al.* 1993).

The cytoplasmic domain of Fas consists of 145 amino acids, in which no motif for enzymic activity such as kinases or phosphatase can be found (Itoh *et al.* 1991). However, about 70 amino acids in this region has significant similarity with a part of the cytoplasmic region of the type I TNF receptor (Itoh *et al.* 1991). TNF has numerous biological functions, including cytotoxic and proliferative activities (Old 1985). Tartaglia *et al.* (1991) have shown that the type I TNF receptor is mainly responsible for the cytotoxic activity of TNF, whereas the type II receptor mediates the proliferation signal. Analyses of various mutants in Fas and the type I TNF receptor indicated that the domain conserved between Fas and the type I TNF receptor is essential for apoptotic signal transduction (Itoh & Nagata 1993; Tartaglia *et al.* 1993).

In addition to the signal-transducing domain, Fas carries an inhibitory domain for apoptosis in the C-terminus. That is, a Fas mutant lacking 15 amino acids from the C-terminus was an up-mutant, in which about ten times less anti-Fas antibody than that required for the wild-type Fas was sufficient to induce apoptosis (Itoh & Nagata 1993). Moreover, in L929 cells, activation of Fas alone (without metabolic inhibitors) was sufficient to induce apoptosis. It is possible that association of inhibitory molecule(s) mentioned above or modification of Fas at this region down-regulates the activity of Fas to transduce the apoptotic signal.

4. FAS LIGAND, A DEATH FACTOR

(a) *Identification and purification of Fas ligand*

Rouvier *et al.* (1993) have established a CTL hybridoma cell line (d10S) which has cytotoxic activity against thymocytes from wild-type, but not from *lpr*-mice, suggesting the presence of a Fas ligand on its surface. We prepared a soluble form of Fas (Fas-Fc) by fusing the extracellular region of Fas to the Fc region of human IgG. The fusion protein inhibited the Fas-dependent CTL activity of d10S cells in a dose-dependent manner, and the Fas ligand was detected by FACS on the cell surface of d10S cells using labelled Fas-Fc (Suda & Nagata 1994). A subline of d10S which abundantly expresses the Fas ligand was established by repeated sorting on FACS. After sorting sixteen times, the subline (d10S16) expressed about 100 times more Fas ligand and showed about 100 times more cytotoxic activity than the original d10S cells. The Fas ligand was then purified from d10S16 to homogeneity by affinity chromatographies using Fas-Fc and Con A. The purified Fas ligand had M_r of 40 kDa, and had cytolytic activity specifically against cells expressing Fas (Suda & Nagata 1994), suggesting that a single protein (Fas ligand) is sufficient to induce apoptosis by binding to Fas.

(b) *Molecular properties of the Fas ligand*

A cDNA library was constructed from the sorted subline of d10S cells, and Fas ligand cDNA was isolated by the panning procedure using mFas-Fc (Suda *et al.* 1993). The recombinant Fas ligand expressed in COS cells could kill the cells expressing

Fas by apoptosis. Its amino acid sequence indicated Fas ligand is a type II membrane protein belonging to the TNF family. As shown in figure 3, members of the TNF family include Fas ligand, TNF, lymphotoxin (LT), and ligands for CD40, CD30, CD27 and 4-1BB. All members of this family are type II membrane proteins except for LTα (or TNFβ) which is produced as a soluble cytokine. When Fas ligand was over-produced in COS cells or d10S16 subline, the soluble form of Fas ligand can be found in supernatant (Suda *et al.* 1993). These results suggest that under abnormal conditions, the soluble form of Fas ligand can be produced in the body as found in the TNF system (Old 1985). The tertiary structure of TNF has been extensively studied. It forms an elongated, antiparallel β-pleated sheet sandwich with a jelly-roll topology (Eck & Sprang 1989; Banner *et al.* 1993; Eck *et al.* 1992). The significant conservation of the amino acid sequence among members suggests that others of the family including Fas ligand, have a structure similar to TNF. However, despite the high similarity of the Fas ligand with TNF (about 30% identity of the amino acid sequence level), Fas ligand does not bind to the TNF receptor (Suda *et al.* 1993).

Northern hybridization analysis of rat tissues indicated that Fas ligand is expressed abundantly in the testis, moderately in the small intestines and weakly in the lung. Whereas, little expression of Fas ligand mRNA was observed in the thymus, liver, heart and ovary where Fas is abundantly expressed. In accord with the expression of Fas ligand in the CTL cell line of d10S, activation of splenocytes with phorbol myristic acetate (PMA) and ionomycin strongly induced the expression of Fas ligand mRNA (Suda *et al.* 1993). However, the expression level of Fas ligand mRNA in thymocytes was relatively weak even after activation with PMA and ionomycin.

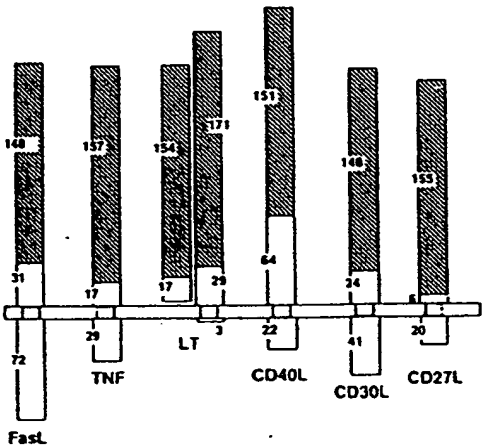

Fig. 3. TNF family. Members of the TNF family are schematically shown. The members include the Fas ligand (FasL), TNF, Lymphotoxin (LT) which consists of LTα and LTβ, CD40 ligand (CD40L), CD30 ligand (CD30L) and CD27 ligand (CD27L). The slashed regions have significant similarity. Numbers indicate the amino acid number of the conserved, the spacer and intracellular regions.

5. PHYSIOLOGICAL ROLES OF THE FAS SYSTEM

(a) *Involvement of the Fas system in development of T cells*

As described above, the Fas gene is the structural gene for *lpr*. Because the mice that are homozygous at *lpr* develop lymphadenopathy and suffer from auto-immune disease (Cohen & Eisenberg 1991), it is clear that Fas plays an important role in the development of T cells. However, it remains controversial at which step of T cell development Fas is involved. T cells are killed by apoptosis at least in three steps during their development (Ramsdell & Fowlkers 1990). In the thymus, T cells carrying T cell receptors which do not recognize self-MHC antigens as a restriction element are killed or 'neglected', whereas the T cells recognizing the self antigens are killed by a process called 'negative selection'. Analysis of thymic T cell development in wild-type and *lpr* mice has suggested that the 'neglected' thymocytes escape from apoptosis in the thymus of *lpr* mice, then migrate to the periphery (Zhou *et al.* 1993). On the other hand, Herron *et al.* (1993) and Sidman *et al.* (1992) reported that the development of T cell in the thymus is essentially normal in *lpr* mice. In addition to the thymus, autoreactive mature T cells are deleted in the periphery (Kabelitz *et al.* 1993). Fas is expressed in activated mature T cells (Trauth *et al.* 1989), and the prolonged activation of T cells leads the cells susceptible against cytolytic activity of anti-Fas antibody (Owen-Schaub *et al.* 1992; Klas *et al.* 1993). Because mature T cells from *lpr* mice are resistant against anti-CD3-stimulated suicide, this suggests a role of Fas-mediated apoptosis in the induction of peripheral tolerance and/or in the antigen-stimulated suicide of mature T cells (Bossu *et al.* 1993; Russell & Wang 1993).

(b) *Involvement of the Fas system in CTL-mediated cytotoxicity*

Fas ligand is expressed in some CTL cell lines and in activated splenocytes (Suda *et al.* 1993), suggesting an important role of the Fas system in CTL-mediated cytotoxicity. Two mechanisms for CTL-mediated cytotoxicity are known (Golstein *et al.* 1991; Podack *et al.* 1991; Apasov *et al.* 1993). The one is a Ca^{2+}-dependent pathway in which perforin and granzymes play an important role. The other pathway is a Ca^{2+}-independent pathway, the mechanism of which is not well understood. It is possible that most Ca^{2+}-independent CTL activity is mediated by the Fas system.

Not only in the thymocytes and lymphocytes, Fas is expressed in other tissues such as the liver, heart and lung (Watanabe *et al.* 1991). Although these organs are rather stable, and no apparent abnormal phenotypes are seen in these tissues of *lpr* mice, Fas may also be

involved in development and/or turnover in these tissues. Because abnormal activation of Fas (administration of anti-Fas antibody) causes severe tissue damage (Ogasawara *et al.* 1993) as described above, it is possible that the Fas system is involved in various human diseases such as fulminant hepatitis. In this regard, it is notable that a particular CTL cell line induces apoptosis in hepatocytes, which leads to fulminant hepatitis (Ando *et al.* 1993; Chisari 1992).

The mutations in Fas (*lpr*) cause lymphadenopathy and autoimmune disease, whereas Fas ligand was found in CTL which kill the tumour cells. These results imply that the Fas/Fas ligand system involved in the T cell development plays an important role in CTL-mediated cytotoxicity. Moreover, it suggests that the killing process of autoreactive T cells in T cell development and the killing process of tumour cells by CTL may proceed by a similar mechanism. As schematically shown in figure 4*a*, autoreactive T cells recognize the self antigens as a complex with MHC which are expressed in the antigen-presenting cells, and may be activated through the T cell receptor. Activation of T cells induces the expression of Fas and Fas ligand, and kill each other. Because *lpr* mice have defects in B cells by producing autoantibodies, the Fas system may also operate to delete the autoreactive B cells in a similar fashion. In the CTL reaction, the target cells such as tumour cells or the cells transformed with virus express the tumour antigen or virus antigen as a complex with MHC. The interaction of CTL with these cells may activate the CTL through the T cell receptor, and induce the Fas ligand gene. The Fas ligand then binds to Fas on the target cells, causing apoptosis (figure 4*b*).

Mice carrying the *gld* mutation show phenotypes similar to *lpr* (Cohen & Eisenberg 1991). From the bone-marrow transplantation experiments between *lpr* and *gld* mice, Allen *et al.* (1990) suggested that *gld* and *lpr* are mutations of an interacting pair of molecules. As shown above, the *lpr* is a mutation in Fas which is the receptor for Fas ligand. Therefore, it is possible that *gld* mice carry mutations in the Fas ligand gene. In fact, we have recently shown that *gld* mice carry a point mutation in the Fas ligand, which inactivates the ability to bind Fas (Takahashi *et al.* 1994).

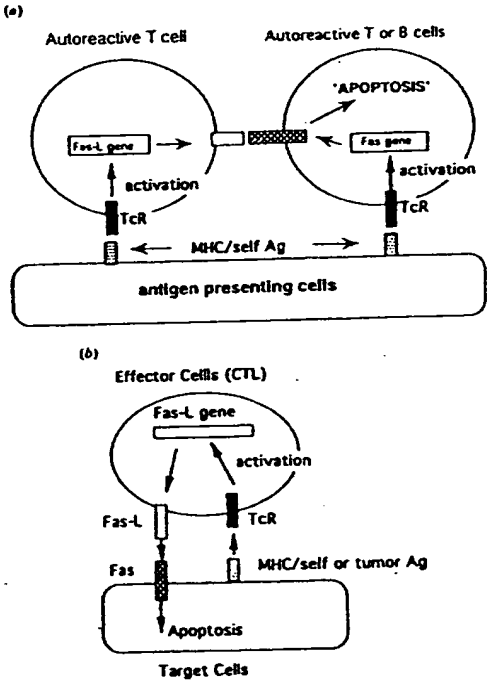

Figure 4. A model for the Fas-mediated cytotoxicity. (*a*) A proposed mechanism for the Fas-mediated peripheral clonal deletion is schematically shown. Antigen-presenting cells express self antigen as complex as MHC, which interacts with T cell receptor in autoreactive T cells and activate them. Activation of T cell induces Fas and Fas ligand gene expression. Interaction of T cells will cause clonal deletion. (*b*) A proposed mechanism for the Fas-mediated cytotoxicity in the CTL system is schematically shown. The target cells express the self, tumour or virus antigen as a complex with MHC, which interacts with the T cell receptor (TcR) on CTL. This interaction activates the CTL, and induces the expression of the Fas ligand (Fas-L) gene. The Fas-L expressed on the cell surface of the CTL then binds to Fas on the target cells, and induces its apoptosis.

6. PERSPECTIVES

We demonstrated that Fas ligand is a death factor, and Fas is its receptor. These results indicate that

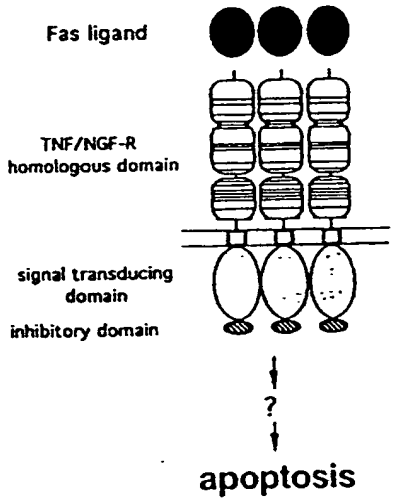

Figure 5. Fas-mediated apoptosis. Fas and the Fas ligand are schematically shown. The Fas ligand binds to Fas on the cell surface probably as a trimer, and activates apoptotic signal transduction. In the cytoplasmic region of Fas, a region of about 80 amino acids is responsible for the signal transduction, while the C-terminal domain (about 15 amino acids) inhibits apoptosis.

just as growth factor and its receptor regulate cell proliferation, apoptosis is also regulated by a death factor and its receptor (figure 5). It would be interesting to examine what kinds of signals are transduced through Fas to induce apoptosis. The gain-of-function mutation of the growth factor system causes cellular transformation, whereas the loss-of-function mutation of the Fas system causes lymphadenopathy. In this regard, Fas and the Fas ligand may be considered as tumour suppressor genes. The loss-of-function mutation in the growth factor system causes the disappearance or dysfunction of specific cells. As pointed out above, abnormal activation (gain-of-function) of the Fas or Fas ligand may cause fulminant hepatitis or other diseases such as CTL-mediated autoimmune diseases. If involvement of the Fas system in human diseases is proven, antagonistic antibodies against Fas or Fas ligand, or the soluble form of Fas, could be used in a clinical setting.

I thank Dr O. Hayaishi and Professor C. Weissmann for encouragement and discussion. The work was carried out in a collaboration with Dr T. Suda, Dr J. Ogasawara, Dr T. Takahashi, Dr M Adachi, Dr N. Itoh and Dr R. Watanabe-Fukunaga, and supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan. I also thank Ms K. Mimura for secretarial assistance.

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